Abstracts

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001 STUDIES ASSESSING PROTEIN S-NITROSYLATION IN THE RAT HEART

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Nitric oxide (NO) has many well-defined effects on the cardiovascular system, including regulation of vascular tone, excitation contraction coupling, and injury during ischaemia and reperfusion. NO mediates many of its biological responses by the well-defined and important role of cGMP production and signalling through protein kinase G. However, in recent years there has been increasing awareness that many of the functional effects of NO may be a result of protein S-nitrosylation, a reversible coupling of NO to a reactive cysteine thiol. A novel method for detecting and purifying S-nitrosylated proteins was recently described, which utilises the ascorbate selective reduction of S-nitrosylated proteins and their subsequent labelling with biotin. The S-nitroso group on the thiol group of cysteine is thus 'switched' for biotin, which allows detection on Western blots probed with streptavidin-HRP and purification on streptavidin-agarose columns.
We utilised this method of measuring protein S-nitrosylation in isolated rat hearts. Preliminary studies with homogenates treated in vitro with the nitric oxide donor (100μM SNAP for 30 min) were used to generate the positive controls. Indeed, when samples treated in vitro with an NO donor were analysed by the biotin-switch method, a number of S-nitrosylated proteins were detected on non-reducing Westerns. Other control experiments, highlight there is a propensity for the spontaneous decomposition of S-nitrosylated proteins, which yield small ascorbate independent signals. Attempts to limit ascorbate independent labelling, which may be due to redox active copper or iron, using the metal chelators Neocuproine (0.2 mM) or DTPA (1 mM) was only partially successful. Currently, we are assessing the ability of the method to detect changes in protein S-nitrosylation in intact isolated hearts administered different classes of NO-donor, as well as patho-physiologically relevant interventions such as ischaemia and reperfusion.

002 MODIFICATION OF CARDIAC PEROXIREDOXIN IN RESPONSE TO HYDROGEN PEROXIDE

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Peroxiredoxins (Prxs) are ubiquitous peroxidase enzymes that help regulate endogenous levels of peroxides throughout the body, including within cardiomyocytes and the vasculature. Peroxiredoxins, like the glutathione peroxidases, are oxidoreductases that reduce both hydrogen peroxide and organic (alkyl) peroxides. Recent data suggest that peroxiredoxins (and glutathione peroxidases) can act as sensitive peroxide sensors, capable of transmitting a non-specific peroxide signal into a precise cysteine-specific modification on the surface of a target protein. Peroxiredoxins in other tissues are known to respond to changes in the prevailing hydrogen peroxide concentration through modification of their peroxide reductase activity, protein structure, and conformation. Using the isolated rat heart as a model, studies have been initiated to map the effects of hydrogen peroxide upon cardiac peroxiredoxins.
Isolated rat hearts were aerobically perfused in the Langendorff mode, followed by perfusion with hydrogen peroxide for 5 min over a micromolar to millimolar concentration range. The total protein content was extracted in the presence of SDS and maleimide (so preventing the further modification of all free cysteines), followed by SDS-PAGE (reducing and non-reducing) and western blotting. Probing the blotted extracts with a range of anti-Prx antibodies shows that the two closely related isoforms, Prx-1 and Prx-2, undergo the most significant changes. Submillimolar concentrations of hydrogen peroxide increase the extent of disulphide bond formation within the Prx homodimers and also initiate hyperoxidation of the peroxidatic catalytic cysteine residue to cysteine sulphinic acid. Although these two processes involve the same catalytic cysteine residue, they do not appear to be mutually exclusive.

003 PROTEOMIC AND METABOLOMIC ANALYSES OF ATHEROSCLEROTIC AORTAS DERIVED FROM APOE-DEFICIENT MICE

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Proteomics and metabolomics are emerging technologies to study molecular mechanisms of diseases. We applied these techniques to identify protein and metabolite changes in vessels of apoE−/− mice on normal chow diet.
Using two-dimensional gel electrophoresis and mass spectrometry, we identified about 80 protein species that were altered during various stages of atherogenesis. Immune activation, redox imbalance and impaired energy metabolism preceded lesion formation in apoE−/− mice. Immunoglobulins were deposited prior to the accumulation of other serum proteins. Oxidative stress in the vasculature was reflected by the oxidation status of 1-Cys peroxiredoxin and correlated to the extent of lesion formation in 12 month-old apoE−/− mice. Nuclear magnetic resonance spectroscopy revealed a depletion of the adenosine nucleotide pool in vessels of 10 week-old apoE−/− mice. Attenuation of lesion formation was associated with alterations of NADPH generating malic enzyme, which provides reducing equivalents for lipid synthesis and glutathione recycling, and successful replenishment of the vascular energy pool possibly serviced by increased glucose utilisation.
Our study provides the most comprehensive dataset of protein and metabolite changes during atherogenesis published so far and highlights potential associations of immune-inflammatory responses, oxidative stress and energy metabolism.

004 ASSOCIATION OF IMMUNE REACTIONS TO OXLDL WITH CHRONIC INFECTIONS AND CAROTID ATHEROSCLEROSIS

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We investigated whether associations exist between immune reactions to oxidised LDL (OxLDL), chronic infections and carotid atherosclerosis as quantified by ultrasound.
We measured IgG and IgM autoantibody titres to copper-oxidised-LDL and malondialdehyde-LDL (OxLDL-AB), IgG and IgM apolipoprotein B-100-immune complexes (ApoB-IC) and titres of antibodies to E coli-lipopolysaccharide (LPS), mycobacterial heat shock protein 65 (mHSP65), C.pneumoniae, H.pylori and cytomegalovirus and evaluated their relationship to cardiovascular risk factors, chronic infections and incident/progressive carotid atherosclerosis in the Bruneck Study. OxLDL-AB and ApoB-IC levels remained stable over time as indicated by strong correlations between 1995 and 2000 measurements ($p<0.001$ each). Significant associations existed between all OxLDL markers and antibody titres to all pathogens, especially to E coli-LPS and mHSP65. These relationships were generally more pronounced for IgG OxLDL-AB and IgG ApoB-IC. Both IgG and IgM for OxLDL-AB and ApoB-IC were significantly elevated in subjects with chronic infection as defined by clinical criteria. Titres of IgG, but not IgM, OxLDL-AB and ApoB-IC inversely correlated with total cholesterol, LDL-cholesterol and apoB concentrations. IgG OxLDL markers were positively and IgM markers were inversely associated with incident and progressive carotid atherosclerosis in univariate analysis but were not independent predictors in multivariate analysis.
Our study provides the first evidence for an association between human OxLDL-AB and ApoB-IC and chronic infections. This is also the first prospective population-based evidence that IgG and IgM OxLDL-AB and

ApoB-IC do not independently predict the presence or absence of early carotid atherosclerosis.

005 ALTERED SUPEROXIDE ANION PRODUCTION AND ANTIOXIDANT GENE EXPRESSION IN FOETAL UMBILICAL ARTERY SMOOTH MUSCLE CELLS FROM PRE-ECLAMPTIC PREGNANCIES

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It is well recognised that endothelial dysfunction in pre-eclampsia (PE) may result from an elevated circulating levels of reactive lipid peroxides and oxygen radicals, the source of which is likely to be the under-perfused placenta. Studies of the feto-placental circulation in PE are infrequently reported, although the feto-placental vasculature and regulation of intracellular Ca2+ are often abnormal (Steinert, *et al. FASEB J* 2003;**17**:307–9). In this study we investigated basal and homocysteine or 4-hydroxynonenal (HNE) stimulated superoxide (O2-.) production and expression of the antioxidant-like enzyme heme oxygenase-1 (HO-1) in human umbilical artery smooth muscle cells (HUASMC) isolated from normal and PE pregnancies. O2- production, assessed by lucigenin chemiluminescence, was elevated 3–4 fold in PE compared to normal HUASMC, and this increase was prevented by pre-treatment of cells with ascorbic acid (100 mM, 24h). HUASMC were challenged with the lipid peroxidation product HNE (10–20 mM, 24h), found in placental tissue of normal and PE women, or the electrophilic agent diethylmaleate (DEM, 100 mM, 24h), and HO-1 expression in normal and PE HUASMC was determined by immunoblotting. Cells were cultured under normal incubator conditions (20% O2, 5% CO2) or physiological oxygen conditions (4–5% O2, 5% CO2). Under 20% O2, DEM and HNE induced HO-1 expression in both normal and PE cells in a dose dependent manner, although HO-1 expression was markedly attenuated in PE cells. Under normoxic O2 conditions, expression of HO-1 in response to DEM and HNE was again lower in PE compared to normal cells. Our findings suggest that HUASMC from PE pregnancies have an increased basal release of O2-. but an attenuated antioxidant defence compared to normal cells.

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006 ANTIOXIDANT GENE EXPRESSION IN OESTROGEN RECEPTOR KNOCKOUT MICE

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Estrogens protect against cardiovascular diseases in pre-menopausal women (Bush, *Ann NY Acad Sci* 1990;**592**:263–71) and have also been shown to reduce atherogenesis in animal models (Mikkola & Clarkson, *Cardiovasc Res* 2002;**53**:605–19). Accumulating evidence suggests that phytoestrogens are protective against vascular diseases (Zhang, *et al. J Nutr* 2003;**133**:2874–8). This study investigates whether phytoestrogens act via oestrogen receptors (ER) to modulate the expression of antioxidant genes such as heme oxygenase-1 (HO-1). Tissues from wildtype or ERα or ERβ knockout mice were harvested for quantitative PCR analyses of antioxidant gene expression. To investigate whether phytoestrogens alter antioxidant gene expression in vascular cells, human aortic smooth muscle cells (HASMC) were treated (24 h, 0.1 & 25 μM) with 17b-estradiol or genistein and expression of HO-1 assessed by immunoblotting. Basal levels of HO-1, glutathione peroxidase (GPx) and MnSOD were higher in livers from female ER$\alpha-/-$ compared to wildtype, but no difference was observed in livers from male wildtype and ER$\alpha-/-$ mice. Male ER$\beta-/-$ mice exhibited 40–50% lower hepatic expression of HO-1, GPx and MnSOD compared to wildtype mice. In livers from female ER$\beta-/-$ only HO-1 levels were lower compared with wildtype mice and this was associated with an increased production of superoxide assessed by lucigenin chemiluminescence. HO-1 expression was significantly elevated in HASMC treated with 25 mM genistein for 24h. These findings suggest that ER-deficient animals have altered antioxidant gene expression, and phytoestrogens may play a role in mediating antioxidant gene expression. Ongoing studies are determining whether antioxidant responses to dietary phytoestrogens are impaired in vascular cells isolated from ER-deficient mice.

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007 TRANSFORMING GROWTH FACTOR-β1 MODULATES HEME OXYGENASE-1 LEVELS AND ENHANCES SUPEROXIDE GENERATION IN HUMAN AORTIC SMOOTH MUSCLE CELLS

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Reactive oxygen species and oxidised low density lipoproteins (oxLDL) contribute to atherogenesis resulting from endothelial and smooth muscle cell (SMC) dysfunction. We have previously reported that oxLDL induces the expression of the antioxidant-like stress protein heme oxygenase-1 (HO-1) in human aortic SMC (HASMC).[1] HO-1 catabolises the pro-oxidant heme to the antioxidants biliverdin and the vasodilator carbon monoxide.[2] Transforming growth factor-β1 (TGF-β1) stimulates vascular SMC growth and extracellular matrix synthesis which contributes to vascular remodelling in atherosclerosis and restenosis. In vascular diseases, antioxidant genes such as HO-1 and elevated levels of the endogenous antioxidant glutathione (GSH) serve as an adaptive response to limit oxidative injury. We have now investigated whether TGF-β1 induces ROS generation to modulate HO-1 and intracellular reduced GSH levels and the involvement of mitogen-activated protein kinases (MAPK) as mediators of HO-1 induction.

Cells were treated with TGF-β1 (0–5 ng/ml, 0–24 h) and HO-1 expression and phosphorylation of p42/p44^{MAPK}, p38^{MAPK} and c-Jun N-terminal kinase (JNK) determined by western blot analyses. GSH levels were measured by fluorescence and superoxide generation assessed by lucigenin chemiluminescence.

TGF-β1 (2.5 ng/ml, 2 h) treatment led to phosphorylation of JNK and p38^{MAPK} but not p42/p44^{MAPK} and increased superoxide production which was attenuated by the NADPH oxidase inhibitor apocynin. TGF-β1 also caused a time dependent induction of HO-1 expression, reaching a maximum between 8–12 h (10.3 ± 2.3 fold, mean$\pm$SE, $n=3$, $p<0.01$). In contrast, TGF-β1 (0–10 ng/ml, 0–24 h) had negligible effects on intracellular GSH levels. Modulation of TGF-β1 mediated free radical production and HO-1 expression may provide mechanistic insights for the contribution of this cytokine to vascular disease processes.

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1. **Anwar**, *et al. Free Rad Biol Med* 2005;**39**:227–36.
2. **Maines**. *Ann Rev Pharmacol Toxicol* 1997;**37**:517–54.

008 THE NEGATIVE INOTROPIC EFFECT OF β_3-ADRENERGIC RECEPTOR STIMULATION IN NNOS$^{-/-}$ MICE IS RESTORED BY OXYPURINOL

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Recent evidence suggests that increased superoxide production by xanthine oxidoreductase (XOR) may decrease bioavailability of eNOS-derived NO in the myocardium of nNOS knockout mice (nNOS$^{-/-}$). To test the functional relevance of these findings, we compared the inotropic and $[Ca^{2+}]_i$ transient response to β_3-adrenergic receptor (AR) stimulation (with BRL-37344, 10 μM in conjunction with the β_1/β_2-AR blocker, nadolol, (NA) 10 μM) in LV myocytes from eNOS$^{-/-}$ and nNOS$^{-/-}$ mice and their wild type controls (WT). BRL+NA caused a significant reduction in cell shortening in WT myocytes ($4.9\pm0.2\%$ *vs.* $5.3\pm0.2\%$ in basal conditions, $p<0.01$) but not in eNOS$^{-/-}$ myocytes. Interestingly, the negative inotropic effect of BRL+NA was also abolished in nNOS$^{-/-}$ mice ($5.7\pm0.2\%$ *vs.* $5.8\pm0.2\%$ in basal conditions, $p=0.55$), despite a 2-fold increase in β_3-AR protein expression and no change in eNOS protein level in the nNOS$^{-/-}$ myocardium (4 hearts/group). Consistent with these results, BRL+NA significantly decreased the amplitude of $[Ca^{2+}]_i$ transients in nNOS$^{+/+}$ (F_{365}/F_{380}: 0.52 ± 0.050 vs 0.59 ± 0.037 in basal conditions, $p=0.02$) but not in nNOS$^{-/-}$ mice (0.64 ± 0.052 vs 0.64 ± 0.046 in basal conditions, $p=0.76$). Inhibition of XOR by 100μM oxypurinol restored the negative inotropic effect of BRL+NA in nNOS$^{-/-}$ mice ($5.5\pm0.4\%$ vs $6.4\pm0.4\%$ in control, $p<0.01$.

Our data suggest that increased superoxide production by XOR in the LV myocardium of nNOS$^{-/-}$ mice abolishes the eNOS-dependent negative inotropic effects of β_3-AR stimulation, presumably by scavenging eNOS-derived NO. These findings suggest that aspects of the functional phenotype of nNOS$^{-/-}$ mice may result from inhibition of eNOS-mediated effects.

009 ALDOSTERONE-INDUCED ACTIVATION OF A NOX2 NADPH OXIDASE PLAYS AN IMPORTANT ROLE IN INTERSTITIAL CARDIAC FIBROSIS

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NADPH oxidases are major cardiovascular sources of reactive oxygen species (ROS). We previously showed that a Nox2-containing NADPH oxidase is critical for the development of angiotensin II (AngII)-induced interstitial cardiac fibrosis. In this study, we investigated the potential role of the Nox2 oxidase in aldosterone (ALDO)-induced fibrosis. Male mice lacking Nox2 (KO) and wild-type littermate controls (WT) underwent unilateral nephrectomy and were then infused with ALDO (0.2 mg/kg/day) together with 1% NaCl, 0.3% KCl in drinking water (ALDO/salt) or vehicle (SHAM) ($n \geq 6$ per group).
Results: After 4 weeks of ALDO/salt, systolic blood pressure had risen to similar levels in both WT and KO mice (WT, 130.0 ± 5.8 mmHg; KO, 134.1 ± 4.6 mmHg, $p<0.05$ compared to their respective SHAM controls). Likewise, there were similar increases in heart/body weight ratio in WT and KO ALDO/salt groups compared to their respective SHAM controls (WT, 4.98 ± 0.13 vs 4.21 ± 0.09 mg/g, $p<0.05$; and KO, 4.97 ± 0.18 vs 4.45 ± 0.08 mg/g, $p<0.05$). Interstitial cardiac fibrosis (Masson's trichrome staining) increased significantly in WT ALDO/salt cf. SHAM ($12.0 \pm 1.7\%$ vs $6.3 \pm 0.3\%$; $p<0.05$) but no increase was observed in KO ALDO/salt ($5.8 \pm 1.0\%$ vs $6.8 \pm 0.8\%$, p=NS). In WT ALDO/salt mice there were significant increases in procollagen I and fibronectin mRNA assessed by real-time RT-PCR (2.1 ± 0.3 and 1.9 ± 0.2 fold respectively cf SHAM; both $p<0.05$) but these were inhibited in KO ALDO/salt mice (1.1 ± 0.1 and 0.9 ± 0.1 fold respectively cf SHAM; both p=NS). Myocardial NAPDH oxidase-dependent superoxide production (lucigenin chemiluminescence) increased by $26.4 \pm 9.9\%$ in ALDO/salt WT mice cf SHAM ($p<0.05$) but was not increased in KO ALDO/salt mice versus SHAM ($2.2\% \pm 5.3\%$, p=NS). NADPH-dependent superoxide production in all groups was inhibited by diphenyleneiodonium and Tiron but not by L-NAME, rotenone or allopurinol.
Conclusion: These results indicate that the Nox2 NADPH oxidase plays a pivotal role in the interstitial cardiac fibrosis induced by ALDO/salt in mice. The effects on interstitial fibrosis in this model are dissociated from the effects on blood pressure and cardiac hypertrophy, which were unaltered in the Nox2 knockout mice.

010 THE ROLE OF A NOX2-CONTAINING NADPH OXIDASE IN POST-INFARCTION CARDIAC REMODELLING

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Reactive oxygen species (ROS) are involved in the processes underlying cardiac remodelling. A Nox2-containing NADPH oxidase is an important source of ROS in the heart. Here, we investigated its role in cardiac remodelling after myocardial infarction (MI) using mice lacking Nox2 (KO) and wild-type littermate controls (WT). Adult female KO and WT mice underwent coronary artery ligation or sham surgery and were studied 4 weeks later. Infarct size measured 24 h post-MI was similar in a cohort of WT and KO mice (45 ± 2 vs $49 \pm 2\%$). Echocardiography indicated significant attenuation of left ventricular (LV) dilatation in KO (eg LV end-diastolic dimension (LVEDD): sham 3.77 ± 0.09, MI 4.91 ± 0.10 mm; $p<0.05$) versus WT (LVEDD: sham 3.77 ± 0.04, MI 5.26 ± 0.12 mm; $p<0.05$) mice at 4 weeks post-MI. Similarly, in vivo assessment of high-fidelity LV pressure (1.4 F Millar catheter) indicated better LV systolic and diastolic function in KO (eg $LVdP/dt_{max}$: sham 11795 ± 839, MI 7862 ± 342 mmHg/s; $p<0.05$) compared to WT ($LVdP/dt_{max}$: sham 11351 ± 303, MI 5746 ± 234 mmHg/s; $p<0.05$) mice after MI. Overall LV/body weight ratio increased to a similar extent in WT and KO mice. However, expression of atrial natriuretic factor mRNA (real-time RT-PCR) was increased to a significantly greater extent in WT MI versus KO MI (1389 ± 233 vs $427 \pm 97\%$; $p<0.05$). Similarly, increases in expression of procollagen Iαl and fibronectin mRNA were significantly greater in WT MI compared to KO MI (443 ± 70 versus $146 \pm 18\%$ and 607 ± 126 versus $210 \pm 42\%$ respectively; both $p<0.05$). Increases in interstitial cardiac fibrosis post-MI (picrosirius red) were significantly greater in WT (5.0 ± 0.4 to $13.5 \pm 1.0\%$; $p<0.05$) compared to KO (5.1 ± 0.3 to $8.8 \pm 0.9\%$; $Pp<0.05$). Mortality at 4 weeks post-MI was not significantly different between groups (WT 21%, KO 8%). These data suggest that a Nox2-containing NADPH oxidase contributes significantly to the processes underlying cardiac remodelling and contractile dysfunction post-MI.

011 MKK3-DEPENDENT SIGNALLING IS NOT REQUIRED FOR LEFT VENTRICULAR REMODELLING FOLLOWING INFARCTION

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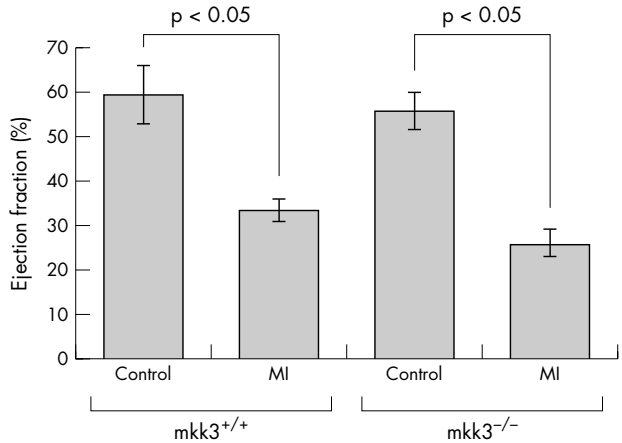
The p38 MAPK (p38) signalling pathway has been implicated in the progression of chronic heart failure and remodelling following MI by a number of independent research groups. The alpha isoform of p38 is thought to mediate injury and is preferentially activated by the upstream kinase MKK3. We examined the role of this kinase using a mkk3-targetted mouse line subjected to permanent coronary artery ligation.
28 days after MI, haemodynamics in male mkk3+/+ (WT) (n=6) and mkk3−/− (KO) (n=6) littermates were assessed using a 1.4F pressure-conductance catheter. In addition, morphometric analysis of infarct and remote areas was performed after fixation at a known end-diastolic pressure. MI groups were compared to un-operated time-matched WT and KO controls (n=5 and n=6 respectively).
We found uniform LV dysfunction after MI which did not differ by genotype despite significantly depressed LV ejection fraction (fig), dP/dt min and max and preload adjusted maximal power (PAMP). On morphometry both MI groups demonstrated significant remodelling with marked LV dilation but all measured parameters were similar in KO and WT mice.
Our results imply that, unlike p38, MKK3 is not required for pathological remodelling after MI suggesting p38 MAPK activation through alternative pathways.



012 NEGATIVE REGULATION OF THE CARDIAC STRESS RESPONSE (HSP72 EXPRESSION) AND BASAL SENSITIVITY TO ISCHAEMIA-REPERFUSION BY PKC EPSILON

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In the heart, HSP72 is a key mediator of the stress and inflammatory responses and plays an important role in mediating heat-stress- and ischaemia-induced tissue protection. Protein kinase C epsilon (PKCε) is also a key mediator of cardioprotection, but its role in regulating the expression of HSP72 has not been fully explored. As expected HSP72 protein expression was induced in vivo in WT mouse hearts 24 hours following repetitive, transient ischaemia/reperfusion (3xI/R). However, in PKCε KO hearts surprisingly HSP72 expression was basally elevated in the absence of stress and KO hearts had significantly smaller infarcts compared to WT hearts at baseline. Also, in isolated neonatal rat ventricular cardiomyocytes (NRVM), overexpression of a dominant negative (dn) PKCε induced HSP72 in the absence of stress whereas wild type (wt) PKCε had no effect. HSP72 expression was induced by simulated ischaemia/reperfusion (sI/R) but induction was unaffected by pharmacological inhibition of PKC. Similarly, basal induction of HSP72 expression was also recapitulated in PKCε-null MEFs and this was reversed by re-expression of wtPKCε. Overexpression of wt PKCε also inhibited HSP72 induction by a sub-maximal heat stress. Furthermore, in NRVM overexpression of wtPKCε repressed hHSF-1-dependent activation of an HSE-dependent reporter gene, suggesting that the repressive

effect of PKCε on HSP72 expression is mediated via HSF-1. In conclusion, PKCε contributes to the maintenance of the stress response in a basally repressed state by inhibition of HSF-1 and interference with PKCε results in de-repression of the stress response in the absence of stress.

013 ACTIVATION OF AKT AND ERK UPON REPERFUSION IS ESSENTIAL FOR DELAYED PHARMACOLOGICAL PRECONDITIONING

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Delayed preconditioning, the phenomenon whereby either transient ischaemia or pharmacological pre-treatment of the heart results in protection against ischaemic injury 24 hours later, has hitherto assumed to be reliant upon de novo protein synthesis and the promotion of a ischaemic-resistant phenotype. However, a number of pro-survival protein kinases are also involved in the preconditioning signalling pathway, but whether these kinases are involved primarily in the trigger phase of preconditioning or in the mediation of delayed preconditioning remains unclear. Using angiotensin II (ATII) as a preconditioning mimetic and a Langendorff mouse model of ischaemia/reperfusion, we tested the hypothesis that ATII pre-treatment (5 μg/kg, ip) would result in preconditioning dependent on the activation of 'reperfusion injury salvage kinases' Akt and Erk upon 30 min reperfusion following 35 min ischaemia. ATII administration resulted in bi-phasic protection against ischaemia/reperfusion injury, with infarct size reduction in the acute phase (19±4% versus control 35±4%), lasting at least 2 hours. By 6 hours this protection was lost (32±2%), to re-emerge 24 hours later (25±4%), and finally waning 96 hours post-treatment. The concomitant determination of Akt, Erk, p38 and Jnk phosphorylation prior to injurious ischaemia revealed little correlation to observed protection. In contrast, at both 1 hour 24 hours after ATII administration there was robust phosphorylation of both Akt and Erk (1 hour: 4.5±0.5 and 1.9±0.6 fold; 24 hours: 24±2 and 2.1±0.1 fold increase of p-Akt and p-Erk over respective controls, $p<0.001$) within 5 min of reperfusion following injurious ischaemia. Moreover, administration of PD98059 or Wortmannin for the first 15 min of reperfusion, respectively inhibiting MEK or PI3K activity prevented the phosphorylation of Erk or Akt and abrogated the protection (35±4% and 33±3% respectively versus controls 33±5% and 33±4%). Thus, we demonstrate that for the first time that kinase activation upon reperfusion triggered is pivotal to delayed pharmacological preconditioning, which may provide fresh avenues for understanding the mechanisms of delayed protection.

014 POST ISCHAEMIC ADMINISTRATION OF A3 ADENOSINE RECEPTOR AGONIST AMELIORATES MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY VIA PI3-KINASE.

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A3 adenosine receptor (A3R) agonist, 2-Cl-IBMECA has previously been reported to limit lethal reperfusion injury through anti-apoptotic and anti-necrotic mechanisms. However, the consequence of activating A3Rs at different time points during reperfusion has not been studied before in a model of myocardial ischaemia reperfusion. Isolated rat hearts were subjected to 35 min left coronary artery occlusion followed by 120 min reperfusion. Treatment groups (n = 5) were perfused with 2-Cl-IBMECA (1 nM), introduced at different time intervals during reperfusion (R), either at start of R, 15 min, 30 min or 60 min after start of R. Wortmannin (5 nM) the PI3 kinase (PI3K) inhibitor was co-perfused during the same time period. Results are show in the table. Hearts underwent triphenyl tetrazolium staining for infarct size assessment, or were frozen for Western blot analysis. The results are summarised in the table. Administration of the 2-Cl-IBMECA (1 nM) at reperfusion, 15 and 30 min after initiation of R led to a significant reduction in infarcted myocardium. No protection was observed when 2-Cl-IBMECA was administered at 60 min post R. The protection afford by 2-Cl-IB-MECA was abrogated by Wortmannin (5 nM). This study is the first to show that A3R activation at various time points post reperfusion can still reduce myocardial injury. These data suggest that introducing A3R agonists post reperfusion may provide an important therapeutic strategy for acute coronary syndromes that involve reperfusion.

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Table 1 Effect of administering A3 agonist at different time points during reperfusion

Group	Reperfusion	15 min	30 min	60 min
Control	54.3±6.6	54.3±6.6	54.3±6.6	54.3±6.6
2-Cl-IB-MECA	32.0±4.0*	23.9±5.4*	24.0±8.9*	60.0±8.5%**

I/R = Mean Infarct/Risk% *, $p<0.05$ vs Control, **, $p<0.05$ vs R, 15 and 30 min

015 MYOCARDIAL INJURY IS REDUCED BY ADMINISTRATION OF CASPASE INHIBITORS AT VARIOUS TIME POINTS DURING REPERFUSION

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Apoptosis contributes significantly to cardiomyocyte death during ischaemia reperfusion (IR) injury. Caspase family proteases play an essential role in the execution of apoptosis. Caspase inhibitors have been found to be cardioprotective if administered before the time of ischaemia or at the start of reperfusion (R). The consequence of administering caspase inhibitors at different time points during R has not been studied before in a model of myocardial IR. Studies were undertaken in isolated Langendorff perfused rat hearts. After stabilisation, hearts underwent 35 min left coronary artery occlusion followed by 120 min R. Treatment groups (n = 5) were perfused with either non selective caspase inhibitor (ZVAD-fmk, 0.1 μM) or caspase-3 inhibitor (Ac-DEVD.cmk, 0.07 μM). The caspase inhibitors were introduced either at start of R, 15 min, 30 min or 60 min after start of R. Hearts underwent triphenyl tetrazolium staining for infarct size assessment, or were frozen for Western blot analysis. The results are summarised in the table. Administration of the caspase inhibitors at R, 15, 30 and 60 min after initiation of R, led to a significant reduction in infarcted myocardium. Western blot analysis examined the effectiveness of the inhibitors at the various time points. This study is the first to show that administration of caspase inhibitors at various time points post R can still reduce myocardial injury. These data suggest that introducing caspase inhibitors post reperfusion may provide an important therapeutic strategy in the clinical settings of ischaemia-reperfusion.

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Table 1 Effect of administering caspase inhibitors at different time points during reperfusion

Group (n)	Reperfusion	15 min	30 min	60 min
Control	54.3±6.6	54.3±6.6	54.3±6.6	54.3±6.6
ZVAD-fmk	17.3±2.0*	30.5%±3.4*	31.6±3.9*	31.5±6.6*
Ac-DEVD-fmk	14.4±3.9*	15.7±3.6*	18.5±2.7*	26.5±5.1*

I/R, Mean Infarct/Risk% *, $p<0.05$ vs Control

016 SUSTAINED INTRACELLULAR ACIDOSIS ACTIVATES MYOCARDIAL ERK VIA THE CLASSICAL RAS-RAF-MEK PATHWAY

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We have investigated the effect of sustained intracellular acidosis on extracellular signal-regulated kinase (ERK) regulation in adult rat ventricular myocytes (ARVM), and determined the mechanism of this regulation. Cultured ARVM were equilibrated in Tyrode solution for 90 min prior to experiments. ARVM were subjected to intracellular acidosis ($pH_i \sim 6.6$) for up to 20 min, by transient exposure to NH_4Cl and its subsequent washout with Tyrode solution containing the NHE1 inhibitor cariporide (to prevent the recovery of pH_i). After the desired duration of intracellular acidosis, the phosphorylation status of ERK and its downstream effector 90 kDa ribosomal S6 kinase were determined by Western blotting. This revealed a time-dependent, transient activation of both kinases by intracellular acidosis, with maximum activation at 3 min and a return to basal levels by 20 min. In subsequent experiments, pH_i was clamped at 7.2–6.5 by exposing cells to different concentrations of NH_4Cl (0–30 mM) for 3 min, followed by washout for 3 min. Increased ERK phosphorylation was observed at pH_i 6.83, which reached a maximum at pH_i 6.64. Acidosis-induced ERK phosphorylation was not prevented by interfering with signalling via PKC isoforms (with

bisindolylmaleimide I; 1 μM), src family kinases (with PP2; 10 μM) or G protein-coupled receptors (by overexpressing RGS4 or the RGS domain of p115RhoGEF), discounting these pathways as upstream mediators of the response. In contrast, inhibition of MEK1/2 (with UO126; 3 μM) or the adenovirus-mediated overexpression of dominant-negative D208A-MEK1 protein prevented the acidosis-induced phosphorylation of ERK, as did inhibition of c-Raf (with GW 5074; 10 μM). Interference with Ras signalling (with farnesyl protein transferase III inhibitor; 50 μM) or adenovirus-mediated overexpression of dominant-negative N17-Ras protein also prevented acidosis-induced ERK phosphorylation. These data suggest that sustained intracellular acidosis activates ERK through the classical Ras-Raf-MEK pathway.
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017 MAPKAPK-2 DOES NOT MEDIATE THE INJURY ASSOCIATED WITH P38-MAPK ACTIVATION DURING MYOCARDIAL ISCHAEMIA

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Activation of the mitogen activated protein kinase (MAPK) p38 has been implicated in lethal myocardial ischaemic injury. So far, the intracellular mediator for its effects is unclear. MAPK activated protein kinase2 (MAPKAPK2;MK2) is one of its downstream p38 substrate which is thought to determine its nucleocytoplasmic transport, stability and cellular distribution. In this study we investigated the contribution of MK2 by using MK2 deficient mice. Langendorff-perfused hearts subjected to 30 min global ischaemia (I) and 2h reperfusion revealed no significant difference in the infact:risk volume ratio between wild type (WT) and knock-out (KO-) hearts. Moreover, the infarct size in both WT and KO was similarly and significantly decreased by 1 μM SB203580, a p38 MAPK inhibitor. Although immunoblotting experiments showed that the protein levels of total and phosphorylated p38 MAPK (T-p38; P-p38, respectively) were lower in control KO (KOC) than WTC mice. There was no significant difference in the ratio of P-p38 to T-p38 in WT and KO after 20 min ischaemia, suggesting that the ratio but not the actual level is the main determinant of ischaemic injury. The cellular distribution of P-p38 was then quantified in 12 μm heart sections using immuno-flourescence Laser confocal microscopy. A significant increase was observed in P-p38 nuclear fluorescence levels in KOC when compared to WTC (KOC 1828 ± 147.2; WTC 1223 ± 5.4, $p<0.01$). Such intensity was similar to both WTI and KOI (WTI 2018 ± 107; KOI 1916 ± 128.9). In conclusion, p38-induced myocardial ischaemic injury is not mediated by MK2. Instead, the high levels of nuclear P-p38 and distinctive T-p38 translocation may predispose to its effects through an alternative p38 substrate, which is yet to be identified.

018 NOVEL ROLES FOR NESPRIN-2 IN STRESS SIGNALLING BETWEEN FOCAL ADHESIONS AND THE NUCLEUS

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Nesprins are a novel family of spectrin repeat containing proteins. They are encoded by two genes and multiple isoforms exist, the largest consisting of a C-terminal transmembrane domain, a central rod like region and an N-terminal paired calponin homology domain. Nesprins were originally identified as markers of human vascular smooth muscle cell differentiation. Isoforms localise to multiple subcellular compartments including focal adhesions and the nucleus and we hypothesised that nesprins may act as multivalent scaffold proteins. In this study we describe novel roles for nesprin-2 in stress signalling between focal adhesions and the nucleus. Using an immunoprecipitation and immunofluorescence approach we identified Hic5 and Erk2 as nesprin-2 binding partners and show that the association and localisation of these proteins are altered upon oxidative stress. Both nesprin-2 and Hic5 translocate from focal adhesions to the nucleus but remain associated whereas nesprin-2 and Erk2 disassociate within the nucleus upon translocation. Preliminary data indicates that nesprin-2 isoforms undergo nucleocytoplasmic shuttling via a CRM-1 mediated nuclear export signal. Taken together these data suggest nesprin-2 plays a pivotal role in stress signalling in human vascular smooth muscle cells by controlling levels of signalling molecules within the nucleus.

019 REGULATION OF P21$^{CIP1/WAF1}$ MRNA AND PROTEIN IN CARDIAC MYOCYTES

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Terminally-differentiated cardiomyocytes, the contractile cells in the heart, are susceptible to oxidative stress that induces apoptosis. The regulation of $p21^{Cip1/Waf1}$ was studied in cardiomyocytes exposed to H_2O_2 (an example of oxidative stress). RT-PCR indicated that $p21^{Cip1/Waf1}$ mRNA was upregulated from 2 h in response to 0.2 mM H_2O_2. $p21^{Cip1/Waf1}$ protein (assessed by immunoblotting) was similarly upregulated despite ~95% inhibition of global protein synthesis, suggesting that $p21^{Cip1/Waf1}$ is regulated translationally and that it constitutes a significant aspect of the apoptotic response. Phosphorylation of $p21^{Cip1/Waf1}$ protein by PKB/Akt increases its stability, but inhibition of the pathway with LY294002 indicated that, in cardiomyocytes exposed to H_2O_2, this was not a significant factor in the upregulation of $p21^{Cip1/Waf1}$. Transfection experiments with luciferase reporter genes indicated that H_2O_2 suppressed the expression of luciferase, whether a constitutive promoter or the promoter for $p21^{Cip1/Waf1}$ was used, providing additional evidence for translational regulation of full-length $p21^{Cip1/Waf1}$ mRNA. Further studies focused on the $p21^{Cip1/Waf1}$ gene. A region was identified within intron 1 of rat, mouse and human $p21^{Cip1/Waf1}$ genes, with high homology, which overlapped with a published EST sequence. This novel sequence was expressed in $p21^{Cip1/Waf1}$ mRNA in cardiomyocytes and, both the novel and classical $p21^{Cip1/Waf1}$ transcripts were upregulated in response to H_2O_2. Further investigations will focus on the precise role of the classical and novel $p21^{Cip1/Waf1}$ transcripts in cardiomyocyte apoptosis.

020 A NOVEL ROLE FOR PROTEIN PHOSPHATASE 2A (PP2A) IN GPCR-MEDIATED REGULATION OF THE SARCOLEMMAL NA^+/H^+ EXCHANGER IN ADULT MYOCARDIUM

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G_q protein-coupled receptor stimulation increases sarcolemmal Na^+/H^+ exchanger (NHE1) activity in cardiac myocytes by an extracellular signal-regulated kinase (ERK)/p90 ribosomal S6 kinase ($p90^{RSK}$/RSK)-dependent mechanism, most likely via RSK-mediated phosphorylation of the NHE1 regulatory carboxyl terminal domain. Adenosine A_1 receptor stimulation inhibits this response through a pertussis toxin-sensitive, G_i protein-mediated pathway, but the distal inhibitory signalling mechanisms remain poorly defined. In cultured adult rat ventricular myocytes (ARVM), stimulation of A_1 receptors by N^6-cyclopentyl adenosine (CPA) inhibited an increase in NHE1 phosphorylation induced by the α_1-adrenoceptor agonist phenylephrine, without affecting activation of the ERK/RSK pathway. CPA also induced significant accumulation of the catalytic subunit of type 2A protein phosphatase ($PP2A_c$) in the particulate fraction, which also contained the cellular NHE1 complement; this effect was abolished by pre-treatment with pertussis toxin. Immunocytochemistry and confocal microscopy imaging of CPA-treated ARVM revealed significant co-localisation of $PP2A_c$ and NHE1, in intercalated disc regions of ARVM. In an in vitro kinase assay, purified $PP2A_c$ dephosphorylated a GST-NHE1 fusion protein containing aa 625–747 of the NHE1 regulatory domain, which had been pre-phosphorylated by recombinant RSK2; this action of $PP2A_c$ was inhibited by okadaic acid and endothall, in a concentration-dependent manner. In intact ARVM, CPA inhibited a phenylephrine-induced increase in NHE1 activity, but this inhibitory effect of CPA was abolished in the presence of a $PP2A_c$-inhibitory concentration of endothall. These studies reveal a novel role for the PP2A holoenzyme in adenosine A_1 receptor-mediated regulation of NHE1 activity in ARVM, the mechanism of which appears to involve G_i protein-mediated translocation of $PP2A_c$ and NHE1 dephosphorylation.

021 THE PHOSPHATASE INHIBITOR, NSC672121, PREVENTS THE INDUCTION OF HYPERTROPHY IN CARDIOMYOCYTES

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The phosphatase inhibitor, 2-(2-mercapto-1-hydroxyethyl)-3-methyl-1,4-naphthoquinone (NSC672121; also known as Cpd5) is a potent inhibitor of cell growth, and is thought mediate the resultant cell cycle arrest by inhibiting CDC25 phosphatases. Recently, it has been shown that NSC672121 treatment also can lead to hyperphosphorylation of

extracellular signal-regulated kinases 1 and 2 (ERK1/2). The mechanism of this hyperphosphorylation has been suggested to involve inhibition of the dephosphorylation of Raf and/or ERK1/2 by CDC25 or mitogen-activated protein kinase (MAPK) phosphatases, respectively. Since MAPK signalling pathways have been associated with the induction of cardiac hypertrophy in response to different hypertrophic stimuli, we have investigated the effect that NSC672121 has upon hypertrophic growth in cardiomyocytes. In this study, we have shown that NSC672121 prevented the induction of hypertrophy by serum in a dose-dependent manner. The sizes of neonatal myocytes treated with serum and 2.5 or 5 μM NSC672121 were reduced significantly, 39±3% and 62±3%, respectively, compared to cultures stimulated with serum alone (100±3%, means±SE, n=3). Similarly, ANF mRNA expression in neonatal and adult myocytes treated with serum and 2.5 μM NSC672121 was significantly reduced to levels equivalent to that of serum-starved cultures. Moreover, NSC672121 did not induce hyperphosphorylation of ERK1/2 in adult myocytes at doses $\leqslant$10 μmol/L, although these doses were sufficient to inhibit significantly the induction of ANF expression, consistent with an abrogation of cardiomyocyte hypertrophy. In conclusion, we have demonstrated that NSC672121 abrogates the serum-induced activation of the Raf-MEK-ERK signalling pathway and blocks the resultant hypertrophic growth response in both neonatal and adult cardiomyocytes. Taken together, phosphatases inhibited by NSC672121, such as CDC25, might prove to be suitable therapeutic targets for the treatment of detrimental hypertrophy.

022 CLONING OF HCB1 & 2, TWO NOVEL CARDIAC-ENRICHED TRANSCRIPTION FACTORS

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The human cardiac troponin I (hTnIc) gene shows both cardiac-specific and developmentally regulated expression. Previous studies have identified an overlapping CACC/Sp1 element in a critical region within hTnIc the promoter and electrophoretic mobility shift analysis identified four proteins present in cardiac muscle extracts, which bind this region. Two of these proteins were identified as Sp1 and Sp3. However, the other two proteins appear to be novel CACC-box binding factors as, unlike all other CACC-box binding factors identified to date, they are cardiac-enriched and their characteristics of DNA binding suggests that they are not zinc finger factors. These novel proteins were named HCB1 and 2 for Heart CACC-box binding proteins 1 and 2. We hypothesise that HCB1 and 2 may therefore be important novel tissue-restricted regulators of cardiac gene expression. In an attempt to clone the cDNA sequences for HCB1 and 2, we have employed yeast one hybrid cloning. A yeast reporter strain with the ''bait'' CACC/Sp1 element cloned upstream of the *HIS3* selectable marker was transformed with a ''prey'' neonatal rat cardiac myocyte cDNA library to trap the cDNA sequences for HCB1 and 2. Plasmid DNA was isolated from positive library clones and sequenced. Initial library screening has yielded 70 potential positive clones. These include cDNA sequences encoding a so far uncharacterised protein as well as Sp1 clones and two leukaemia related zinc finger factor clones. An initial bio-informatic expression analysis of the novel protein suggests that it is expressed in the heart but not in skeletal muscle. Current work is aimed at examining the expression pattern of potential candidate clones and comparing binding characteristics with endogenous HCB1 and 2 proteins. Other molecular and cellular techniques will then be used to establish whether HCB1 and 2 represent a new class of cardiac-enriched transcription factors. HCB1 and 2 may therefore have the capability to be phosphorylated and thus could play an important role in signalling pathways such as the MAPK (mitogen-activated protein kinases) pathway in the cardiac myocyte.

023 CALCINEURIN AND PROTEIN KINASE C REGULATE CYCLOOXYGENASE-2 EXPRESSION IN THE HEART

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Upregulation of components of the acute inflammatory response, such as COX-2 and iNOS, is important for the adaptation of the myocardium to ischaemia-reperfusion stress. The aims of this study were to determine (1) if the calcineurin signalling pathway regulates cyclooxygenase-2 (COX-2) expression in the heart, (2) if it interacts with the protein kinase C (PKC) pathway and, (3) if it is cell-type specific. Adenovirally mediated overexpression of an active mutant of calcineurin Aα (CnAα) in cultured neonatal rat ventricular cardiomyocytes (NRVMs) induced COX-2 expression. COX-2 induction by CnAα was potentiated by co-expression of the wild type α or ε isoforms of PKC. Reporter constructs comprising +104 to −1796 bp of the human COX-2 gene proximal promoter region confirmed the effect of CnAα and PKC which was mediated by a region between −1796 and −170 containing a number of NFAT and AP-1 sites. In contrast, no COX-2 induction was seen in isolated cultured adult cardiomyocytes under the same experimental conditions. Therefore, COX-2 expression in NRVMs was assessed by immunofluorescence confocal imaging in relation to the myocyte-specific marker α-actinin. COX-2 was basally expressed in all cardiac cells but strong COX-2 induction was only observed in non-myocytes (fibroblasts) when cells are overexpressing CnAα+PKCα or CnAα+PKCε. In addition, studies using frozen mouse heart sections showed higher COX-2 expression only in myocardial endothelial cells of mice treated i.p. with LPS 5 mg/Kg. In conclusion, these results demonstrate that the calcineurin signalling pathway mediates COX-2 induction in cardiac cells that the effect is potentiated by PKC and that integral myocardial non-myocytes play an important role in mediating the cardiac inflammatory response.

024 CALCINEURIN ISOFORMS IN STRIATED MUSCLE REGENERATION

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The calcium/calmodulin-dependent serine/threonine phosphatase calcineurin plays a central role in skeletal and cardiac muscle hypertrophy and regeneration. In response to increased calcium, calcineurin induces dephosphorylation, nuclear translocation and activation of NFAT, a process sensitive to the action of the immunosuppressive drug Cyclosporin-A. Calcineurin (Cn) consists of a catalytic (CnA) and a regulatory (CnB) subunit. CnA isoforms are composed of a catalytic domain, a CnB-interacting domain, a calmodulin-binding region and an autoinhibitory domain, which maintains the enzyme inactive in the absence of increased calcium. Three CnA genes have been described: CnAα and CnAβ are ubiquitously expressed, whereas CnAγ is expressed in brain and testis. We and others have described a naturally occurring CnAβ splicing variant lacking the autoinhibitory domain (termed CnAβ1, as opposed to the predominant CnAβ2 isoform), although its function remains obscure. Here, we show that CnAβ1 expression decreases throughout heart development and is transiently induced during skeletal muscle regeneration, whereas CnAβ2 expression changes in an opposing fashion. Transgenic over-expression of CnAβ1 in post-mitotic skeletal muscle enhances muscle regeneration, reduces fibrosis and induces a faster resolution of inflammation. In contrast, mice over-expressing a full version of CnAα show increased fibrosis and inflammation and delayed muscle regeneration. In uninjured animals, CnAβ1 induces a fast-to-slow fiber switch, accompanied by IKKα activation and extensive NFATc dephosphorylation, compared both to wild type and to CnAα-expressing mice. In conclusion, CnAβ1 represents a unique physiological form of calcineurin with physiological and molecular properties distinct from other CnA isoforms. The ability to CnAβ1 to enhance cardiac muscle regeneration is currently under investigation.

025 METABOLIC REMODELLING IN HUMAN PERSISTENT ATRIAL FIBRILLATION AS REVEALED BY AN INTEGRATED PROTEOMIC AND METABOLOMIC ANALYSIS

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Atrial fibrillation (AF) leads to several different forms of remodelling processes within the atria, which contribute to its self-perpetuating nature. The exact mechanisms underlying atrial remodelling are as yet not fully understood.
To assess the remodelling processes in persistent AF, we analysed protein and metabolite changes in human atria, which were fibrillating or in sinus rhythm, by using 2D gel electrophoresis and nuclear magnetic resonance spectroscopy (NMR). Firstly, structural remodelling of the cytoskeleton was evident on 2D gels as fragmentation of cytoskeletal components namely actin, a compensatory increase in microtubules and alterations in desmin and small heat shock proteins. Secondly, oxidative stress during AF was reflected in the depletion of the anti-oxidant peroxiredoxin 1. Thirdly, enzymes involved in energy metabolism were upregulated, including 3-oxoacid transferase, the key enzyme for

ketolytic energy production. Consistent with our proteomic findings, NMR analysis revealed a rise of beta-hydroxybutyrate in AF, which was accompanied by increased concentrations of tyrosine, glycine, leucine and a tendency towards accumulation of lactate. Interestingly, the cellular energy pool (adenosine pool, total creatine) was maintained but phosphocreatine, a marker of the cellular energetic state, was significantly elevated during persistent AF.
This study is the first to utilise a combination of proteomic and metabolomic techniques on human myocardial tissue to demonstrate that persistent AF represents a hypermetabolic state reflected by a rise in phosphocreatine and serviced by increased ketone body utilisation. This ''metabolic remodelling'' may be a key element contributing to the self-perpetuating nature of this resilient arrhythmia.

026 TEMPORAL CHANGES IN THE IGF-I/IGFBP-1 AXIS IN A MURINE MODEL OF DIET-INDUCED OBESITY AND INSULIN RESISTANCE

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Introduction: Insulin, insulin-like growth factor-I (IGF-I) and IGF binding protein-1 (IGFBP-1) are thought to have complimentary roles in the regulation of glucose homeostasis. Observational studies suggest that patients with insulin resistance and type II diabetes have lower levels of circulating IGF-I. Lower levels of IGF-I and IGFBP-1 have also been implicated in the development of CVD and raising levels of IGFBP-1 may help protect against this increased risk. We have characterised the longitudinal changes in IGF-I, IGF-I sensitivity and IGFBP-1 expression in dietary induced obesity in mice and assessed the effects of IGFBP-1 overexpression on this phenotype.
Methods: Wildtype(WT) mice received an obesogenic or standard chow diet from weaning (n = 6–16). Morphometrics were recorded and insulin sensitivity was assessed by intraperitoneal insulin tolerance tests. Fasting IGF-I levels were measured by ELISA. Hepatic IGFBP-1 expression was measured by real-time PCR. Subcutaneous IGF-I tolerance tests (0.2 mg/g) were performed at similar time points. Transgenic(TG) mice were also fed the obesogenic diet and underwent insulin tolerance testing. Body weight, fat pad mass and fat cell size were measured.
Results: WT mice receiving a high-fat diet had a higher body weight, larger fat pad mass and fat cell size than those fed chow diet. Insulin resistance was increased at 4 and 8 weeks. The hypoglycaemic effect of IGF-I was also significantly diminished in mice fed an obesogenic diet at 8 weeks. IGF-I levels were found to be significantly higher in this group. There was no difference observed in hepatic IGFBP-1. Compared to WTs, TG mice had significantly increased response to insulin after 8–12 weeks high-fat feeding (41.8% decrease in blood glucose at 30 min vs 32.3%, $p<0.02$) but similar body weights and fat depots.
Conclusion: These data, contrary to previously reported observations suggest that IGF-I levels increase as insulin sensitivity decreases and there is an accompanying decrease in IGF-I sensitivity. IGFBP-1 expression in the liver remains unchanged however overexpression of IGFBP-1 protects against the development of insulin resistance despite the development of obesity. This work was supported by the British Heart Foundation.

027 IGF-1 TRANSCRIPTION IN CARDIAC MYOCYTES AND FIBROBLASTS: RELEVANCE TO HEART FAILURE

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Insulin-like Growth Factor 1 (IGF-1) is known to be important for cardiac myocyte growth and survival. IGF-1 increases cardiac output and contractility, reduces afterload and has beneficial effects on the failing heart in vivo. Cardiac myocytes in culture respond to IGF-1 with an increase in rate of protein synthesis and transgenic mice over-expressing IGF-1 or the IGF-1 receptor in the heart develop physiological-type hypertrophy. In spite of these observations surprisingly little is known of how IGF-1 expression is regulated at the transcriptional level in the heart. We recently demonstrated that IGF-1 mRNA expression is elevated in patients who recover from heart failure following left ventricular unloading using an assist device and pharmacological therapy including the beta2 agonist clenbuterol. Here, we have investigated the independent contribution of mechanical unloading and clenbuterol on IGF-1 in rat in vivo, determined the contribution of myocytes and fibroblasts to overall IGF-1 production and investigated the regulation of the IGF-1 promoter. IGF-1 mRNA was induced 6-fold following mechanical unloading (heterotopic transplant model) but unaffected by clenbuterol in vivo. IGF-1 mRNA was present in both cardiac myocytes and fibroblasts in vitro, but was significantly higher in fibroblasts. IGF-1 levels increased with time in culture in myocytes deprived of serum and in senescent cultures with or without serum. IGF-1 promoter-*luciferase* constructs revealed that a region between −1000 and −250 upstream of the E1 promoter plays a significant role in regulating transcription. This region contains a putative binding site for FOXO transcription factors, implicating the Akt/FOXO signalling pathway in IGF-1 regulation. In conclusion, our data show that the IGF-1 gene is responsive to a variety of stress signals in the heart including unloading, serum deprivation and senescence, and indicate that transcriptional mechanisms involving FOXO factors may play an important part in its regulation.

028 MYOCARDIAL EXPRESSION OF THE ARGININE:GLYCINE AMINOTRANSFERASE (AGAT) GENE IS ELEVATED IN HEART FAILURE AND NORMALISED FOLLOWING POST-LVAD RECOVERY: POTENTIAL IMPLICATIONS FOR LOCAL CREATINE SYNTHESIS

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Introduction: Analysis of genetic changes that occur during recovery from heart failure following mechanical unloading using a left ventricular assist device (LVAD) and pharmacologic therapy may provide insight into mechanisms and potential treatments of heart failure. Preliminary microarray analysis of myocardial samples from 6 patients taken before and after recovery showed a significant decrease in myocardial AGAT gene expression during recovery from heart failure. This was unexpected as this rate-limiting enzyme in the creatine synthesis pathway is more commonly associated with kidney and liver. Here we examine in detail AGAT expression and activity in an extended patient series as well as in experimental models of failure.
Methods: Myocardial AGAT mRNA expression was measured by quantitative (Taqman) RT-PCR in implant and explant samples from patients on combination therapy and who recovered (n = 12), as well as end-stage heart failure (ESHF) samples from patients undergoing transplantation without LVAD support (n = 10) and donor hearts with normal hemodynamic function (n = 8). AGAT enzyme activity was determined using a sensitive LC/MS-based assay.
Results: Myocardial AGAT mRNA expression was significantly elevated in LVAD recipients and ESHF patients vs donors (4.3-fold and 2.7-fold respectively, $p<0.002$). In recovery, AGAT mRNA was significantly downregulated (0.23-fold, $p=0.001$) at explant, returning to normal levels. AGAT expression levels correlated inversely with ejection fraction ($R=-0.57$, $p=0.01$) and positively with pulmonary capillary wedge pressure ($R=0.68$, $p<0.0008$) in individual patients. Significant levels of AGAT enzyme activity were detected in normal human donor myocardium (1.7 ± 0.3 nmol/min/g wet weight) and elevated AGAT mRNA levels were seen in a rat model of heart failure(1.3-fold, $p<0.05$).
Conclusion: Our data highlight local regulated expression of AGAT in the myocardium and suggest a specific response to heart failure involving elevated local creatine synthesis. These findings have implications both for the management of recovery patients undergoing combined LVAD and pharmacologic therapy and for heart failure in general.

029 INTEGRIN α7 IS REQUIRED FOR NORMAL HEART STRUCTURE

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Normal heart morphology is dependant on cellular interactions between the extracellular matrix, specific cell surface receptors (integrins), the cytoskeleton, and intrinsic gene regulation. As $\alpha7\beta1$ integrin is the principal integrin-type receptor for laminin in cardiac and skeletal muscle, we investigated the structural role of $\alpha7$ in the heart, by studying the cardiac phenotype of $\alpha7$ null-mutant mice ($\alpha7^{-/-}$).
$\alpha7^{-/-}$ mice have a shortened life span and gross histological analysis of their hearts revealed a reduction in the horizontal-axis and an abnormal elongated structure compared with age matched controls. By 6 months M-mode echocardiography demonstrated a significantly shorter left ventricular end-diastolic diameter ($\alpha7^{-/-}$:3.01 ± 0.14 mm, wt:3.88 ± 0.1 mm; n = 8; $p<0.001$), and a significantly thickened diastolic interventricular septum ($\alpha7^{-/-}$:0.92 ± 0.05 mm;

wt:0.80±0.02 mm; n=8; p<0.03). In addition, a significant decrease in left ventricular diameter/length ratio was noted on long-axis echocardiography ($\alpha7^{-/-}$:0.61±0.03 mm; wt:0.76±0.06 mm; n=6; p<0.001). The structural changes of the left ventricle were accompanied by significantly impaired stroke volume ($\alpha7^{-/-}$:41.97±2.64μl; wt:23.80±2.11μl; n=8; p<0.001). Cellular examination of sectioned hearts demonstrated a marked increase in cardiomyocyte diameter and circumference (c) ($\alpha7^{-/-}$:c=52.8±5μm; wt:c=36.5±3μm; n=3; p<0.01), furthermore, electron micrographs from 12 month old $\alpha7^{-/-}$ and wt hearts revealed profound abnormalities in $\alpha7^{-/-}$ hearts with accumulation of mitochondria into dense regions, fibrosis and lipofuscin granulation. Interestingly, the heart weight: tibia length ratio at 12 months were significantly reduced ($\alpha7^{-/-}$:6.69±0.11 mg/mm; wt:8.58±0.77 mg/mm; n=12; p<0.05). In conclusion, ablation of the α7 gene leads to a severely abnormal elongated structure of the heart. Notably, whilst this involves cardiomyocyte hypertrophy, the reduction in heart weight suggests overall cell loss. This work places α7 integrin as a key player in the maintenance of normal heart structure.

030 MATRIX METALLOPROTEINASE (MMP) AND TISSUE INHIBITOR OF MMPS (TIMP) GENE EXPRESSION IN MYOCARDIAL RECOVERY FROM HEART FAILURE

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Matrix metalloproteinases (MMPs) and the tissue inhibitors of MMPs (TIMPs) play a key role in pathological myocardial remodelling. However, their role in recovery from heart failure (HF) has not been investigated. Here, we studied twelve patients who recovered from end-stage HF following left ventricular assist device (LVAD) support and a pharmacological regime which included unconventional use of the β_2-adrenergic agonist, clenbuterol (combination therapy). We hypothesised that the MMP and TIMP profile would be modified during the reverse remodelling which accompanies the transition to recovery. mRNA levels of MMP-1 to -14 and TIMP-1 to -4 were measured by real-time PCR in left ventricular samples collected at LVAD implant, explant and one-year post-explant. None of the MMPs altered with recovery. In contrast, TIMP-2 was elevated and TIMP-4 reduced at explant compared to implant. At one-year post-explant, TIMP-1 and TIMP-2 were reduced compared to explant. TIMP-3 was unchanged in recovery. The respective contributions of mechanical unloading and clenbuterol were examined using rat models. The results indicate that unloading induces TIMP-1 and TIMP-2 expression whilst clenbuterol represses TIMP-3 and TIMP-4 expression. In conclusion, altered expression of TIMPs, but not MMPs, is a feature of myocardial recovery from HF. Mechanical unloading and clenbuterol have opposing effects on ventricular TIMP expression and induce parallel expression profiles to the recovery process. The data provide insight into the recovery process which will benefit the future refinement of LVAD therapy.

031 SCA-1$^+$ PROGENITORS DERIVED FROM EMBRYONIC STEM CELLS DIFFERENTIATE INTO SMOOTH MUSCLE CELLS WHICH ARE SENSITIVE TO APOPTOSIS

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Background: One of the most promising areas in basic research today involves the use of embryonic stem (ES) cells, because they have the ability to differentiate into somatic cells of all tissue types that can be used for tissue engineering and repair of damaged organs. However, a procedure for producing a large number of smooth muscle cells (SMCs) from ES cells with high purity is still lacking, and little is known about the molecular mechanism of differentiation in these cells.
Methods and results: In the present study, mouse ES cells were cultivated on collagen-IV-coated flasks, and Sca-1$^+$ progenitor cells were isolated with magnetic beads. The isolated cells were placed into gelatine-coated flasks and cultured in DM medium with or without PDGF-BB (10 ng/ml) for 3 to 90 days. Both immunostaining and FACS analyses revealed that more than 95% of these cells were positive for smooth muscle alpha-actin (SMA), SM22, calponin, and smooth muscle myosin heavy chain (SMMHC). Compared with SMCs derived from the aortic wall, Sca-1$^+$ cell-derived SMCs displayed a lower ability of cell proliferation and higher sensitivity to apoptosis as identified by annexin/PI labelling and viability assays. Western blot analysis and caspase-2 activity assays demonstrated that apoptosis of ES-derived SMCs was caspase-2 dependent, but p53 independent. Furthermore, apoptotic cells were significantly decreased (32.12% vs 7.96%, p<0.001), and strikingly, the SMA$^+$ cells increased (65.92% vs 75.43%, p<0.01), when Sca-1$^+$ cells were treated with PDGF-BB plus Z-VDVAD-fmk (20μM), the caspase-2 inhibitors.
Conclusions: Thus, we have established a method for SMC differentiation from ES cells, which could serve as a cellular source for vascular tissue engineering, and demonstrated that these SMCs are sensitive to apoptosis, which can be calibrated by inhibition of caspase-2 activity.

032 TRANSFER OF STEM CELL-DERIVED ENDOTHELIAL CELLS RETARDED NEOINTIMAL LESIONS IN THE INJURED ARTERY

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Background: Embryonic stem (ES) cells have the ability to differentiate into somatic cells of all tissue types, which can be used for tissue engineering and repair of damaged organs. However, no successful procedure for producing a large number of endothelial cells (ECs) from ES cells with high purity is available, and little is known about the therapeutic potential for these stem cell-derived ECs in repairing injured artery.
Methods and results: Mouse ES cells were cultivated on collagen-IV-coated flasks, and Sca-1$^+$ progenitor cells were then isolated with magnetic beads. The isolated cells were cultured in differentiated medium containing 10 ng/ml vascular endothelial growth factor ($VEGF_{165}$) for 6 to 21 days. RT-PCR, immunofluorescence or Western blot analysis revealed that these cells expressed a full range of EC linage-specific markers, including CD31 (PECAM-1), CD34, CD62E (E-selectin), CD105, CD106 (VCAM-1), CD109, CD141, CD144 (VE-cadherin), CD146, FLK-1 (VEGFR2), Flt-1 (VEGFR1), Sca-1, Tie-1/2, and von Willbrand factor. Flow cytometry analysis confirmed that 98.7% of these cells were CD31-positive, and almost all the cells were DiI-acLDL uptake. When Sca-1$^+$ progenitor-derived ECs mixed with Matrogel were subcutaneously implanted into mice, various vessel-like structures were observed. Furthermore, when these ECs infected with adenovirus-LacZ were transplanted into injured mouse artery, they were found to form neo-endothelium that covered the injured areas (86%±13.6%). this cell transplantation resulted in a significant decrease in neointima lesions 2 weeks after injury (8,036 $\mu m^2 \pm 866 \mu m^2$ versus $30{,}026 \mu m^2 \pm 2500 \mu m^2$, p<0.001).
Conclusions: We conclude that Sca-1$^+$ progenitor cells derived from ES cells can differentiate into functional ECs, which accelerate re-endothelialisation of injured artery and reduce neointimal formation. These results indicate that these ECs may serve as a promising cellular source for vascular tissue engineering and cell therapy for vascular diseases.

033 HYPOXIC PRECONDITIONING PROMOTES PROLIFERATION OF MESENCHYMAL STEM CELLS IN VITRO AND DOES NOT ALTER THEIR EFFECTS IN THE INFARCTED RAT HEART IN VIVO

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Rationale: Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent progenitors, perhaps providing a cell source capable of repairing diseased heart tissue. Hypoxic areas are common to both ischaemic heart tissue and the bone marrow but little is known about the maintenance and response of MSCs to hypoxia.
Hypothesis: Hypoxia treated MSCs would improve cardiac function after myocardial infarction compared with normoxic cells.
Methodology: Rat MSCs were isolated from the bone marrow by adherence to plastic and characterised for expression of specific cell surface markers. The cells were cultured for two passages and transduced with a lentiviral vector system expressing GFP. Cells were then exposed to either normoxia (21% oxygen) or hypoxia (1.5% oxygen) for 24 h before injection into the infarct border zone of rat hearts 10 min after permanent coronary artery ligation. Cardiac function was monitored using cine MRI at 1, 4, 10 and 16 weeks post-infarction. In addition, cDNA array hybridisation was carried out on MSCs subjected to normoxia or hypoxia in order to identify molecular pathways involved in cell maintenance and growth.
Results: Rat MSCs exposed to hypoxia for 24 h had a 1.5-2-fold greater proliferation rate than those exposed to normoxia. Significant increases in cardiac output were observed in both treatment groups compared to control animals and GFP positive cells were detected in the infarct

region. A total of 231 mRNAs in MSCs were regulated by short exposures to hypoxia, with a significant proportion of these genes being involved in cell growth, proliferation or survival.
Conclusions: We demonstrate MSCs proliferate better ex vivo in hypoxia than normoxia and these cells improve cardiac function to a similar degree. Hypoxia may be useful in increasing MSC numbers prior to infusion and may influence MSC survival in hypoxic areas of the heart.

034 MRI TRACKING OF POST-INFARCT MYOCARDIAL MACROPHAGE INFILTRATION

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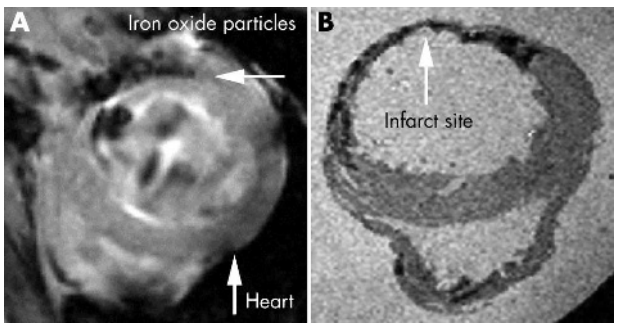
Infarction-induced cardiac macrophage infiltration can lead to chronic inflammation and contribute to heart failure. We have used non-invasive in vivo magnetic resonance imaging (MRI) to determine the timecourse of macrophage migration. Wistar rats were subjected to left coronary artery ligation and macrophage infiltration was monitored by T_2* weighted MRI at days 3 and 7 post surgery. Ultra small super-paramagnetic iron oxide particles (250 μmol Fe/kg bodyweight) were injected 24 hours before imaging, to be taken up by blood monocytes, which homed to the infarct site and differentiated into macrophages. Regions of hypointensity, indicating infiltration, were seen in infarcted hearts and not in those from sham-operated or control animals. Peak macrophage infiltration occurred 7 days after infarction, shown by a large signal void in the 7 day MR image (fig 1). The presence of iron oxide particles in the myocardium was confirmed by high resolution MR microscopy (fig 2). At days 1 and 2 post surgery, levels of C-reactive protein were elevated in both infarcted and sham-operated animals whereas Troponin-I was elevated, and hearts were swollen, in infarcted hearts only. We conclude that contrast enhanced MRI can identify infarcts, visualise the scale of infarction and follow macrophage infiltration over time.



(A) T_2* weighted MR image showing macrophage infiltration 7 days post-infarct. (B) MR micrograph of the same infarcted heart showing iron oxide particles.

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035 IN VIVO TRACKING OF MESENCHYMAL STEM CELLS IN THE INFARCTED RAT HEART BY MAGNETIC RESONANCE IMAGING

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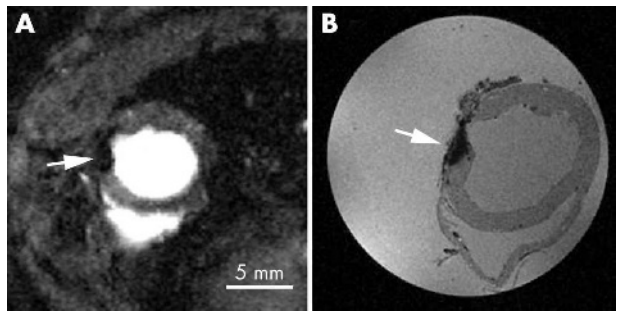
A promising, novel approach to the treatment of myocardial infarction and prevention of heart failure is cell grafting in the damaged myocardium. Several studies have shown that stem cells genetically marked with GFP integrate into the heart, but these cell tracking methods require tissue sectioning. MRI was used to non-invasively track stem cells over 16 weeks and yield information about cell location at various times after injection.
Methods: Rat mesenchymal stem cells (rMSCs) were transduced with lentiviral vectors expressing GFP and labelled with iron oxide particles. Ten minutes after coronary artery ligation, 5×10^5 rMSCs were injected into the infarct periphery (n=13). Rat hearts were repeatedly imaged using cine MRI at 1, 4, 10 and 16 weeks post infarct. At 16 weeks hearts were dissected, fixed and imaged ex vivo using high resolution 3D MR microscopy.
Results: Signal voids caused by the iron oxide particles in the rMSCs were detected in all rat hearts at all times (fig A). MR microscopy identified hypointense regions at the same position as those found in vivo (fig B). Hearts were sectioned and GFP expressing stem cells were found at the locations identified by MR.
Conclusion: MRI can be used to track the migration of iron oxide labelled rMSCs in the infarcted rat heart for at least 16 weeks after injection and MR microscopy can be used to aid tissue sectioning by accurately identify regions of cell engraftment.



036 PROTEOMIC ANALYSIS REVEALS HIGHER DEMAND FOR ANTIOXIDANT PROTECTION IN EMBRYONIC STEM CELL-DERIVED SMOOTH MUSCLE CELLS

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It has been reported that embryonic stem cells (ES cells) can differentiate into vascular smooth muscle (SMCs), but associated changes in protein expression remain to be characterised.
Murine ES cells (ATCC, ES-D3) were pre-differentiated to stem cell antigen-1 positive (Sca-1^+) cells and isolated by magnetic cell sorting. After PDGF-BB (10 ng/ml) treatment for 3 days, 95% of cells expressed SMC markers. Protein extracts of ES cells, Sca-1^+ progenitor cells, ES cell-derived SMCs and aortic SMCs were separated by two-dimensional gel electrophoresis. 300 protein species of each cell line were analysed by mass spectrometry. Protein maps are available on our website http://www.vascular-proteomics.com. Besides decreased expression of cytoskeleton proteins, ES cell-derived SMCs displayed increased oxidation of redox-sensitive proteins and showed higher levels of reactive oxygen species. While immunoblotting revealed an upregulation of numerous antioxidants in ES cell-derived SMCs, enzymatic assays demonstrated lower glutathione concentrations compared to aortic SMCs despite a 3-fold increase in glutathione reductase activity. Moreover, depletion of glutathione by diethylmaleate or inhibition of glutathione reductase by carmustine resulted in a remarkable loss of cell viability in ES cell-derived SMCs.
We present the first protein profiles of murine aortic SMCs, ES cell-derived SMCs and their progenitors. Our comparison revealed that differentiation of ES cells to SMCs is associated with increased oxidative stress and ES cell-derived SMCs require additional antioxidant protection for survival. These results indicate that treatment with anti-oxidants could be beneficial for tissue repair from ES cells.

037 ISCHAEMIC BURDEN AND CIRCULATING ENDOTHELIAL PROGENITOR CELLS: THE IMPACT OF PERCUTANEOUS CORONARY INTERVENTION

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Background: Circulating endothelial progenitor cells (EPCs) increase after acute myocardial ischaemia. However, their response to chronic ischaemia is unknown. We hypothesised that removal of ischaemic burden by percutaneous coronary intervention (PCI) results in a decreased number of circulating EPCs.
Methods: 26 patients with stable exertional angina, undergoing elective PCI for single vessel coronary disease were studied. All subjects had reversible ischaemia on a Bruce protocol treadmill test and/or had a myocardial fractional flow reserve (FFR) <0.75. Circulating EPCs were identified as CD133, VEGFR2 and CD34 positive by FACS. Expression of CXCR4 (the receptor for the chemokine SDF-1) was assessed for homing capability. EPCs and ischaemic burden were reassessed

3–6 months following PCI. Continuous variables were compared using a Wilcoxon Signed Rank test. Data are expressed as mean ± SD.
Results: Follow-up is complete in 17 patients, 137 ± 51 days after PCI. All patients were asymptomatic at follow-up and treadmill exercise testing was negative in all cases. There were no instances of further revascularisation. Clinical measures FFR and treadmill duration (mins) improved significantly from 0.51 ± 0.17 to 0.83 ± 0.09 ($p<0.01$), and 6 ± 2.5 to 7.2 ± 2.8 ($p=0.03$), respectively. $CD133^+$circulating cell numbers were significantly increased from 0.01 ± 0.01 to 0.06 ± 0.06 ($p=0.02$), as were other antigenically determinable subsets of EPCs, $CD133^+VEGFR2^+$ cells from 0.009 ± 0.006 to 0.04 ± 0.03 ($p=0.03$) and $CD34^+CD133^+VEGFR2^+$ cells from 0.003 ± 0.002 to 0.03 ± 0.02 ($p=0.08$). Similarly, the expression of CXCR4 increased following PCI, $CXCR4^+$cells alone increased from 7.5 ± 6.7 to 11.1 ± 9.2 ($p=0.09$), and $CXCR4^+CD133^+$ cells rose from 0.006 ± 0.016 to 0.05 ± 0.04 ($p<0.01$).
Conclusion: Contrary to prediction, the number and homing capacity of EPCs increases following relief of chronic ischaemia. This increase may be due to decreased consumption or improved mobilisation from bone marrow of EPCs following PCI.

038 ENDOTHELIAL PROGENITOR CELLS: A PRIMARY DETERMINANT OF CORONARY COLLATERAL SUPPLY?

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Background: The severity of coronary stenosis is the most consistent predictor of collateral supply to the distal myocardium. However, a coronary collateral circulation exists in some individuals with normal coronary arteries. We hypothesised that collateral supply depends on intrinsic angiogenic factors, such as endothelial progenitor cells (EPCs), particularly in the absence of ischaemia.
Methods: 31 patients with stable single vessel coronary disease were enrolled at elective percutaneous coronary intervention (PCI). Collateral flow index (CFI) was calculated as $(P_d-P_v)/(P_a-P_v)$ by measurement of aortic, central venous and distal coronary pressures (P_a, P_v, P_d) during balloon occlusion. Stenosis was characterised by quantitative coronary angiography (QCA) and fractional flow reserve (FFR). Peripheral blood mononuclear cells were isolated with density centrifugation and $CD133^+$ cells (EPCs) identified by FACS. CFI, QCA and FFR were reassessed 6 months post PCI. Data is expressed as mean ± SD.
Results: Coronary lesion diameter stenosis was 84 ± 15% at baseline and 41 ± 21% at follow up ($p<0.001$). Pre-treatment FFR was 0.51 ± 0.17 and at follow-up was 0.84 ± 0.08 ($p<0.001$). CFI at baseline was 0.23 ± 0.13, falling to 0.14 ± 0.07 at 6 months ($p=0.001$). $CD133^+$ cells at baseline were 0.09 ± 0.05% of all cells. At the time of PCI, CFI was strongly related to coronary diameter stenosis ($r=0.61$, $p<0.001$) and FFR ($r=-0.56$, $p=0.001$) and weakly related to circulating EPC number ($r=0.42$, $p=0.08$). However, 6 months following PCI, CFI was unrelated to diameter stenosis ($r=0.06$, $p=0.83$), weakly related to FFR ($r=-0.49$, $p=0.03$) but strongly related to baseline EPCs ($r=0.87$, $p=0.005$). In a multiple regression model incorporating baseline CFI, baseline EPC number and 6 month FFR, baseline EPC number and baseline CFI remained independently predictive of CFI at 6 months ($p=0.02$ for each parameter).
Conclusion: In the absence of a significant coronary stenosis, circulating endothelial progenitor cells are a powerful determinant of the extent of collateral supply.

039 VASCULAR DYSFUNCTION IN YOUNG ADULT INDIAN ASIANS IS CHARACTERISED BY INSULIN RESISTANCE AND REDUCED ENDOTHELIAL PROGENITOR CELLS

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Introduction: Indian Asians have a higher incidence of coronary artery disease (CAD) than Caucasians. Vascular function, biochemical profiles and levels of circulating endothelial progenitor cells (EPCs) were examined as potential aetiological factors in defining the excess risk.
Methods: 24 Indian Asian and 25 Caucasian age-matched healthy men (mean age 25 years) without known risk factors for CAD were studied. Conduit vessel function was assessed by brachial artery flow mediated dilatation (FMD)(endothelium-dependent) and GTN dilatation (endothelium-independent). Resistance vessel function was assessed by forearm blood flow (FBF) responses to brachial artery infusions of ACh, to estimate endothelium-dependent dilatation and LNMMA (a nonselective NOS inhibitor) to estimate basal NO production. EPCs were quantified by flow cytometry as CD34, CD133 and KDR triple positive cells.
Results: BMI, WHR, BP, lipid profile, fasting glucose and hsCRP in the two groups were not significantly different. Indian Asians had significantly higher fasting insulin levels (6.0 ± 0.7 vs 3.6 ± 0.4, $p=0.005$) and insulin resistance indices (1.3 ± 0.2 vs 0.8 ± 0.1, $p=0.005$). FMD was significantly lower in Indian Asians (6.9 ± 0.3 vs 8.5 ± 0.4, $p=0.003$), as was FBF response to ACh (7.2 ± 0.7 vs 9.6 ± 0.9, $p=0.04$) and basal NO production (0.8 ± 0.1 vs 1.3 ± 0.2, $p=0.03$). GTN dilation was not significantly different. EPC number was significantly lower in Indian Asians (0.046 ± 0.005 vs 0.085 ± 0.009, $p=0.001$) and correlated with FMD in Asians ($r=0.45$, $p=0.05$). On multivariate analysis race was the only independent predictor of FMD and EPC count.
Conclusions: Healthy Indian Asians are more insulin resistant and have lower circulating EPCs than corresponding Caucasians. These abnormalities may contribute to the vascular dysfunction in Asians observed in both conduit and resistance vasculature.

040 CORONARY FLOW RESERVE IS A POOR MARKER OF MICROVASCULAR RESPONSE

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Background: Coronary flow reserve (CFR) is a commonly used non-specific index of coronary microvascular function and is derived from the ratio of maximal hyperaemic (often achieved with a non-specific vasodilator, adenosine) to basal coronary flow. However, comprehensive assessment of the coronary microvascular bed requires information on both endothelium-dependent/–independent responses.
Methods and results: A thermodilution technique (using an intracoronary pressure wire) was employed to sequentially compare CFR (hyperaemia achieved with adenosine 140 μg/kg/ml via a femoral vein) with% change in coronary flow in response to the endothelial agonist substance-P (endothelium-dependent response - 20 pmol/min intracoronary infusion for 2 min) in 40 unobstructed coronary arteries of patients undergoing angioplasty to an adjacent vessel. The mean age of subjects studied was 64 ± 9 years (55% male and 25% diabetic). There was no significant correlation between CFR and endothelium-dependent microvascular responses ($r=0.1$, p=NS). We then studied the relationship between CFR and endothelium-dependent microvascular response, and clinical markers of endothelial dysfunction. There was a strong correlation between endothelium-dependent microvascular response and patient's Framingham Risk Score (FRS - a surrogate marker for cardiovascular risk-factor clustering, hence an indirect measure of endothelial dysfunction – $r=-0.51$, $p<0.001$), but no correlation between CFR and FRS ($r=0.01$, p=NS). Diabetic patients had significantly greater endothelium-dependent microvascular dysfunction than non-diabetics ($p=0.008$). CFR was not influenced by diabetes in this patient cohort (p=NS).
Conclusion: Our results indicate that adenosine derived CFR may not adequately interrogate the endothelium-dependent component of the coronary microvasculature. We propose that information on both endothelium-dependent responses and CFR are required for a comprehensive assessment of the coronary microvascular circulation.

041 MARKERS OF CARDIOVASCULAR RISK IN THE METABOLIC SYNDROME

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Background: C-reactive protein (CRP) is a marker of future cardiovascular (CVS) risk and its levels are elevated in subjects with metabolic syndrome (MS - defined by ATP-III criteria). The association between other markers of CVS risk such as endothelial dysfunction (ED - an early functional disturbance in development of atherosclerosis) and alternative markers of inflammation and MS remain unclear.
Methods & results: We screened 100 (61 male, mean age (years) with MS 36 ± 9, without MS 33 ± 8; p=NS) asymptomatic, non-diabetic, non-smokers for MS and divided subjects into groups depending on presence/absence of MS. All females were premenopausal. Subjects with MS had significant ED ($p<0.001$ - measured by brachial artery flow mediated dilatation (FMD)) and higher levels of tumour necrosis factor α (TNFα; $p<0.001$), interleukin-6 (IL-6; $p<0.001$) and as previously shown CRP ($p=0.002$). There was no correlation between FMD and brachial artery diameter ($r^2=0.01$, p=NS), confirming that arterial size did not influence endothelial reactivity. MS did not influence endothelium-independent vascular function (measured by

glyceryltrinitrate-induced brachial artery dilatation). Oneway ANOVA demonstrated a significant negative linear trend between FMD ($F=21.88$, $p<0.001$) and a positive trend between logTNF ($F=12.93$, $p<0.001$), logIL-6 ($F=28.16$, $p<0.00$) and logCRP ($F=19.01$, $p<0.001$) and the number of ATP-III metabolic criteria present in each subject. Using a logistic regression model the presence/absence of MS was the only independent predictor of ED ($B=2.38$, $SE(B)=0.94$, $p=0.01$).
Conclusion: Markers of CVS risk (ED and systemic inflammation) were significantly higher in asymptomatic subjects with MS within an age range not normally associated with vascular disease. The extent of ED and inflammation increased with the presence of each additional ATP-III metabolic criteria. Presence/absence of MS was the only independent predictor of ED.

042 RAPID PRESSURE-WIRE DERIVED ASSESSMENT OF CORONARY ENDOTHELIUM-DEPENDENT MICROVASCULAR FUNCTION

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Background: Invasive clinical assessment of endothelial-dependent coronary microvascular function currently relies on intracoronary Doppler flow measurements and quantitative coronary angiography (QCA) to derive% changes in coronary blood flow from baseline in response to endothelial agonists. However, this method can be technically challenging with poor reproducibility. We hypothesised that changes in coronary flow derived by a thermodilution method, using the pressure wire (which can function as an intracoronary dual pressure-temperature sensor), could also be used to reliably assess coronary microvascular endothelial function. The transit time (Tmn) of a bolus of room temperature saline using the latter technique is known to be inversely proportional to coronary flow.
Method: 20 patients undergoing angioplasty to a single vessel were recruited and adjacent coronary vessel free of significant disease studied. We compared the% change in absolute coronary flow from baseline using Doppler/QCA with the% reduction in Tmn using thermodilution in response to a 2 min intracoronary infusion of substance P (20 pmol/min).
Results: There was a close correlation ($r=0.76$, $p<0.001$) between% change in absolute coronary blood flow in response to substance P (measured by Doppler Flow Wire) and reduction in Tmn (thermodilution - measured with pressure wire). Bland-Altman analysis revealed a mean absolute difference of 18 ± 19% between the measurements, with 75% of cases having a difference of <20%.
Conclusion: Thermodilution is a simple and reliable technique for assessment of endothelium-dependent microvascular function and can be readily applied in routine practice.